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Species-specific transcriptomic responses in *Daphnia magna* exposed to a bio-plastic production intermediate☆

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ABSTRACT

Hydroxymethylfurfural (HMF) is a plant-based chemical building block that could potentially substitute petroleum-based equivalents, yet ecotoxicological data of this compound is currently limited. In this study, the effects of HMF on the reproduction and survival of *Daphnia magna* were assessed through validated ecotoxicological tests. The mechanism of toxicity was determined by analysis of transcriptomic responses induced by exposure to different concentrations of HMF using RNA sequencing. HMF exerted toxicity to *D. magna* with an EC_{50} for effects on reproduction of 17.2 mg/l. HMF exposure affected molecular pathways including sugar and polysaccharide metabolism, lipid metabolism, general stress metabolism and red blood cell metabolism, although most molecular pathways affected by HMF exposure were dose specific. Hemoglobin genes, however, responded in a sensitive and dose-related manner. No induction of genes involved in the xenobiotic metabolism or oxidative stress metabolism pathway could be observed, which contrasted earlier observations on transcriptional responses of the terrestrial model *Folsomia candida* exposed to the same compound in a similar dose. We found 4189 orthologue genes between *D. magna* and *F. candida*, yet only twenty-one genes of those orthologues were co-regulated in both species. The contrasting transcriptional responses to the same compound exposed at a similar dose between *D. magna* and *F. candida* indicates limited overlap in stress responses among soil and aquatic invertebrates. The dose-related expression of hemoglobin provides further support for using hemoglobin expression as a biomarker for general stress responses in daphnids.

1. Introduction

Modern society is highly dependent on chemical production processes and its products. The usage and production of many of these products, however, may be associated with environmental and human health risks. Production processes are, in addition, often dependent on non-renewable fossil carbon sources. The unsustainability of many products and production processes led the proposition of the so-called 'Green Chemistry' (Anastas and Kirchhoff, 2002) in which products and production processes are designed in such a way that hazards to the environment and human health are limited or absent. Many concepts of the 'Green Chemistry' have become more common in several industrial activities and fields of sciences (Li and Anastas, 2012; Sheldon, 2007), yet the transition from the current chemical industry to sustainable industry is still in its initial stage.

A major challenge is the development of plastics based on renewable sources. Current plastic production is based on petroleum-based building blocks including terephthalic acid (TPA). A promising green alternative for TPA is 2,5-furandicarboxylic acid (FDCA) (Harmsen et al., 2014). FDCA can be polymerized with monoethylene glycol (MEG) to polyethylene 2,5-furandicarboxylate (PEF), a bio-based plastic with comparable properties as conventional PET but with lower greenhouse gas emissions (Eerhart et al., 2012). FDCA can be synthesized from hydroxymethylfurfural (HMF), which is formed through oxidation of fructose and is also occurring in certain processed foods (Murkovic and Bornik, 2007).

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Although compounds like HMF can be produced from plant material, this does not mean that green alternatives are less toxic to organisms than petroleum-based compounds. In fact, FDCA is known to be more toxic to soil invertebrates compared to TPA (Chen et al., 2016).

Essential for the transition to a sustainable bio-based industry is therefore to critically evaluate newly emerged technologies from an ecotoxicological perspective. In fact, some studies indicate that implementation of bio-based productions systems can have adverse environmental effects such as increased carbon releases from soils (Cherubini and Strømman, 2011; Searchinger, 2010) and increased eutrophication (Weiss et al., 2012). In addition, when crops for biomaterials compete for land with crops for food production, this could also affect food prizes (Johansson and Azar, 2007; Searchinger, 2010). Hence, it remains necessary to test the hazards and indirect effects of new bio-based technologies and its products, in order to prevent future negative impacts on human health and the environment (Bluhm et al., 2012; Heger et al., 2012).

This study aims to document ecotoxicological effects of HMF in the fresh water ecosystem. For this purpose, *Daphnia magna*, an important and well-studied fresh water zooplankton species, was used as a model system. In order to enhance our mechanistic understanding of HMF toxicity, we also analyzed the transcriptomic responses induced by HMF in our model species through RNA sequencing. Furthermore, we studied the conservation of stress responses among invertebrates by comparing the transcriptomic responses of *D. magna* with that of the springtail *Folsomia candida*. It was expected that HMF was toxic to *D. magna* and that HMF exposure induced pathways involved in the metabolism of xenobiotics and oxidative stress.

2. Materials and methods

2.1. Test organism and culture conditions

Daphnia magna strain has been cultured for several years at the Vrije Universiteit Amsterdam, and kept in transparent plastic containers with 'Aachener Daphnien medium' (ADaM) (Klüttgen et al., 1994) at 20 °C under a 16/8 h light/dark-regime. Three times a week, media were refreshed and the daphnids were fed with an algae mixture of live *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* cells in a ratio of 3:1 (1.5 mg C/l for approximately 20 individuals/l).

2.2. Test compound, toxicity testing and data analysis

Hydroxymethylfurfural (HMF) (Fig. 2B) (>99% purity) was supplied by BIRD Engineering, the Netherlands. The HMF concentrations tested are listed in Tables S–1. Acute toxicity of HMF was determined according to OECD test no. 202 (OECD, 2004) with concentrations between 10 and 105 mg HMF/l. Five replicate 100 ml vials were filled with 50 ml test solution and each received five daphnids of <24 h old. After 24 and 48 h of exposure, immobility was scored. An additional acute toxicity test was done using 10-day old animals, also following OECD test no. 202 (OECD, 2004) using a concentration range between 20 and 468 mg HMF/l. The effect of HMF on reproduction was assessed using OECD test no. 211 (OECD, 2012) using a concentration range between 0.4 and 33.2 mg HMF/l. Per treatment ten replicate 100 ml vials were filled with 50 ml test solution, each replicate containing one daphnid. The chronic toxicity tests lasted for 21 days. Three times a week, media were refreshed, daphnids were fed and reproductive output per parental animal and parental survival were scored. Juveniles were removed in order to prevent them from competing with parental animals for food. During the experiment, 1 ml medium was sampled at 0 h, 2 and 3 days after preparation for three replicates in order to measure HMF concentrations. Medium samples were stored at −80 °C until measurements.

Toxicity data were analyzed in R version 3.2.0 (http://www.r-project.org), calculating dose response models and EC_{50} values using the 'drc' package (Ritz and Streibig, 2005) and chronic effect levels and confidence interval were determined with log-logistic models. To compare two acute toxicity test results, analysis of variance (ANOVA) using the 'drc' package was conducted. The significance of differences in the number of days until first brood were calculated using a Kruskal-Wallis one-way ANOVA.

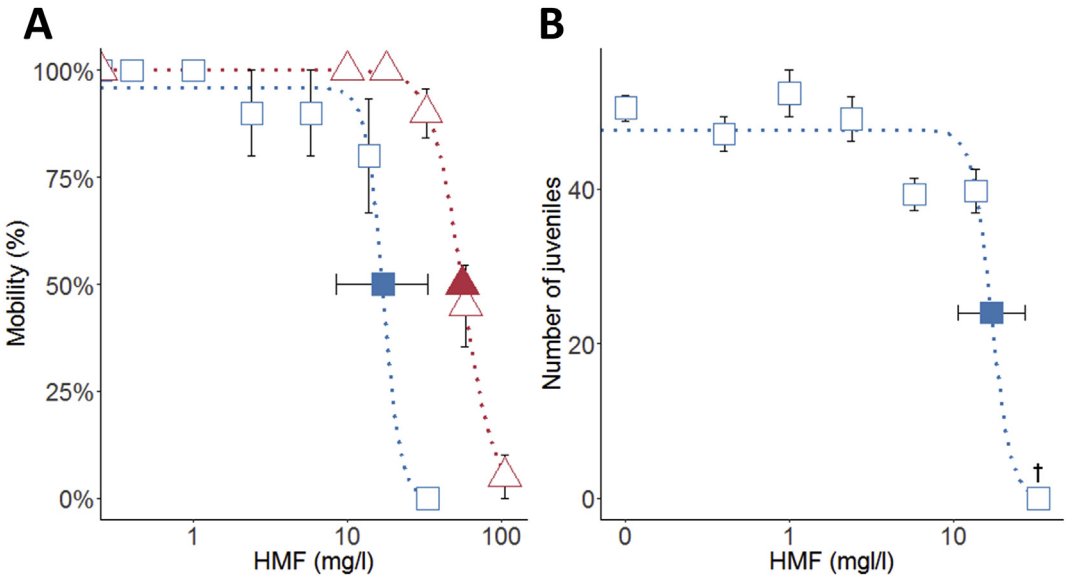

Fig. 1. The effects of HMF on mobility and reproduction. (A) Average mobility (±SE) of *Daphnia magna* exposed to HMF for 48 h (red triangles) and 21d (blue squares) (B) Average reproduction (±SE) of *D. magna* exposed to HMF for 21 days. Dotted lines indicate the log logistic fits. The EC_{50} values (±CI) are is plotted as filled squares. † indicates that all individuals at the relevant concentration died before the end of the experiment. Confidence intervals of the 48 h EC_{50} based on mobility are too small to show. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

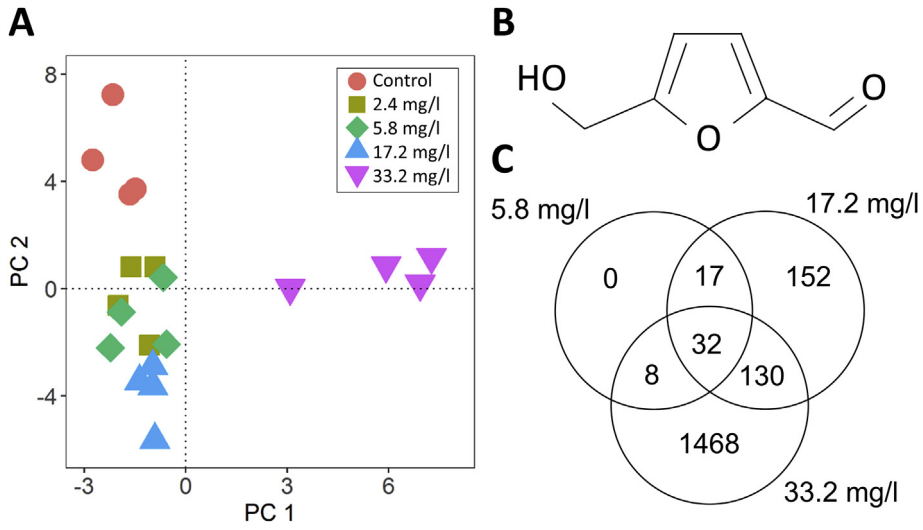

Fig. 2. (A) First two axis of the principal component analysis (PCA) on the counts per million of all of significantly expressed genes in *Daphnia magna* exposed to different concentrations of HMF. PCA is based on a correlation matrix. Proportion of total variance of PC1 and PC2 equals 0.50 and 0.14 respectively. Different points indicate ordination of the different samples. (B) Structure formula of hydroxymethylfurfural (HMF). (C) Overlap in significantly expressed genes in the three highest test concentrations.

2.3. Measurements of HMF in water

To assess the degradation over time of HMF in water, a times series experiment was conducted. Five vials filled with 50 ml AdAM medium at a concentration of 17.2 mg/l HMF. At 0 h, 1, 2, 5, 8, 12 and 15 days after preparation 1 ml medium was sampled and stored at −80 °C until measurements.

Medium samples were diluted in a 5 mM ammonium formate pH 5 buffer and an aliquot of 10 µl was injected into an Agilent 1200 Series LC system (Palo Alto, CA, USA) coupled with an Agilent 6410 electro spray interface (ESI) operating in the positive ion mode prior to triple-quadrupole mass spectrometric detection. The separation was achieved by applying a Biphenyl column (Kinetex, 2.6 µm, 100 Å, 100*2.1 mm) with a gradient of 5 mM ammonium formate pH 5 buffer and methanol. For the quantification the ion transition m/z 127 − m/z 109 was used with the ion transition m/z 127 − m/z 81 for the qualification. HMF measurements were performed at the Department of Environment and Health, Vrije Universiteit Amsterdam. The half-life of HMF was calculated by fitting an exponential curve ($C = C_0e^{-kt}$) through the time series and the half-life $T_{1/2}$ was estimated as $T_{1/2} = \ln 2/k$, where the degradation rate constant k was estimated by least squares regression.

2.4. Transcriptome assay

In order to assess transcriptome-wide effects of exposure to HMF, daphnids were exposed for 2 days to 6 concentrations of HMF, which were chosen based on the outcome of the chronic toxicity assay. Test concentrations included a negative control, the no observed effect concentration (NOEC), the lowest observed effect concentration (LOEC), the calculated EC_{50}, the highest concentration tested in the chronic toxicity assay and a positive control based on the EC_{90} of the test with 10-day old animals (Tables S–1). Per treatment five replicate 100 ml vial were filled with 80 ml test medium, and each received ten 10-days old daphnids. After 48 h, the exposure was stopped by pouring the medium containing the daphnids over a BD Falcon™ 100 µm nylon Cell Strainer filter. Surplus water was discarded. Animals were sampled without removal of the brood pouch. Daphnids were transferred into a 1.5 ml Eppendorf tube using a spatula, snap frozen in liquid nitrogen and stored at −80 °C until RNA isolation. Animals from the positive control were discarded. Average mortality in the positive control was 40% (±SE 19%) after 48 h, thereby validating the test.

2.5. Sample preparation and illumina sequencing

Total RNA was isolated using the Promega SV Total RNA Isolation System according to the manufacturer's protocol. The quantity of the isolated RNA was determined using a Thermo Scientific NanoDrop 2000 spectrophotometer and an Invitrogen Qubit® 2.0 Fluorometer. Integrity of the isolated RNA was evaluated by gel-electrophoresis and by using an Aligent 2100 Bioanzalyer using a Eukaryote Total RNA Nano chip. Per treatment four samples were selected and prepared for sequencing using the Illumina TruSeq® RNA Library Preparation Kit v2 according to the manufacturer's protocol. Quality and quantity were assessed using an Aligent 2100 Bioanalyzer using a DNA 7500 chip. To remove large fragments and short adaptor sequences, cleanup steps were conducted according to recommendations of the manufacturer. Samples were diluted to 10 nmol/l cDNA and subsequently pooled. Samples were sequenced at the VUmc Amsterdam using Illumina HiSeq 2500. The sequencing data was deposited to NCBI's accession number BioProject PRJNA476102.

2.6. Transcriptomic data analysis

Quality of reads and identification of overrepresented sequences was assessed using FastQC. Bases with a quality score lower than 20 at both ends of the reads and overrepresented sequences were removed using trimmomatic v0.33 (Bolger et al., 2014). Reads smaller than 40 base pairs were discarded. The remaining reads were aligned to the reference transcriptome of *D. magna* (NCBI reference number GGRO00000000 under BioProject PRJNA476102) using bowtie2 (Langmead and Salzberg, 2012). The reference transcriptome was derived by RNA sequencing of daphnids exposed to zinc for 2 days. Assembly of the reference transcriptome was done using Trinity RNA-seq (Haas et al., 2013) using default parameters as described in De Boer et al. (2018). Annotation of the reference transcriptome was preformed using Blast2GO pro version 4.1 as described by De Boer et al., 2018. Quantification of the abundances of the sequences was performed using eXpress v1.5.1 (Roberts and Pachter, 2013). Only genes that showed at least one

count per million in at least three samples were included for further analysis. All subsequent data analyses were performed in R. Differential gene expression analysis was conducted using the package 'edgeR' (Robinson et al., 2010). Normalization of the samples was done using the mean of M-values approach (Robinson and Oshlack, 2010). Differential gene expression was performed by applying a generalized linear model likelihood ratio test. In order to assess general transcriptional patterns a heat map of the significant differential expressed genes was made and clustering analysis was conducted using the package 'gplots'(Warnes et al., 2015). In order to test which biological processes were significantly affected, per treatment the significantly regulated genes were subjected to gene-enrichment analysis using the 'topGO' package (Alexa et al., 2006). Statistical testing of significantly enriched GO terms was done using the 'elim' procedure (Alexa et al., 2006). In order to visualize the output of the gene-enrichment analysis, the significant enriched GO terms were subjected to REVIGO analysis, a clustering method that relies on semantic similarity measures (Supek et al., 2011). Log2 normalized counts of the significantly expressed genes were calculated in order to study dose-response functions.

2.7. Cross species transcriptomic comparisons

Ortholog clusters shared between the reference transcriptomes of *F. candida* and *D. magna* transcriptomes were predicted using OrthoMCL version 1.4 as described in (Faddeeva-Vakhrusheva et al., 2016). We used transcriptome data from a previous study in which the springtail *F. candida* was exposed to a HMF concentration with a comparable level of sub-lethal effect causing 50% decrease of reproduction (EC50) (Chen et al., 2016). As the transcriptomic data of the two species were generated on two different platforms, the effect sizes differed. Therefore, standardization was performed using the 'decostand' function in the 'vegan' package (Oksanen et al., 2017). The data sets of the two species were merged after this standardization step, and general patterns of gene expression were assessed by principal component analysis using the 'rda' function in 'vegan'. Subsequently, redundancy analysis was executed to statistically test whether the species and exposure to HMF could explain the transcriptomic responses of the orthologous genes. For this purpose, dummy variables for the two factors were created and included as explanatory variables in the redundancy analysis again using the 'rda' function in 'vegan'.

3. Results and discussion

3.1. Toxicity of HMF to Daphnia magna

Hydroxymethylfurfural affected both the mobility and reproduction of *D. magna* (Fig. 1) with 48 h and 21d EC_{50} values for effects on immobility of 55.3 mg/l (95% CI: 50.3–60.9 mg/l) and 21.4 mg/l (95% CI: 6.3–73.2 mg/l), respectively. The 21d EC_{50} for effects on reproduction was 17.2 mg/l (95% CI: 10.7–27.4 mg/l). Although the 48 h EC_{50} for effects on juveniles was higher than that for 10-day old animals (86.6 mg/l, CI 45.0–148 mg/l), no significant differences between the two log-logistic models were found ($F(1, 45) = 3.257$, $p = 0.078$). For the reproduction data, the model fit was strongly affected by the mortality of all animals at the highest concentration after only four days. The 21d EC_{50} value is, thus, an effect level on population growth. Mortality of a single animal was observed in both the 2.4 mg/l and 5.8 mg/l treatments after 19 days, but since mortality did not show a dose-related pattern these samples were not excluded from the analysis. Exposure to HMF significantly affected the number of days until first reproduction ($X^2(1, 59) = 12.43$, $p = 0.0294$). Animals in the highest concentration produced offspring for the first time on average 1.3 days later than animals in the control (Tables S–2). Aborted eggs, a measure of stress in daphnids, were only observed in the two highest test concentrations but not quantitatively assessed. Daphnids in the two highest concentrations were notably smaller in the first week but no significant differences between treatments in size after 21 days were observed ($F(5, 60) = 0.318$, $p = 0.58$).

HMF was toxic to daphnids, affecting both mobility and reproduction. Comparable effect levels were found in a bioassay similar to OECD test no. 202 (Hessov, 1975). Toxicity increased over time as reflected by the more than two-fold difference between the acute (55.3 mg/l) and chronic EC_{50} (21.4 mg/l) based on mobility. Based on the acute EC_{50}, HMF can be classified under REACH legislation as chronic category 3 for aquatic toxicity (European Union, 2008).

The stability of HMF varied with complexity of the aquatic system. Degradation of HMF was slow in the daphnid medium, with a half-life of 42 days. However, in the presence of daphnids and algae, the degradation was much faster, especially at lower concentrations (Tables S–3). The increased break down capacity at lower HMF concentrations, and enhanced degradation in the presence of other biota could suggest that microbial activity enhances the degradation of HMF in aquatic media. In fact, many microbes are known to be able to degrade HMF (Wierckx et al., 2011). HMF was rapidly degraded in non-sterile soils, while under sterile conditions hardly any degradation took place, confirming a role of microbial degradation in soil (Chen et al., 2016).

The environmental risk of a chemical is not only determined by its toxicity but also by its degradation rate and the risk of exposure. HMF is an intermediate in the production of FDCA. The transformation efficiency of HMF to FDCA is high, up to 97% (Koopman et al., 2010). HMF is thus contained in bioreactors and remainders of the process are biodegraded in contained compartments, the risk of exposure of aquatic ecosystems to HMF seems limited. In case of spills, HMF could potentially be easily degraded by microorganisms under suitable conditions. However, degradation rates in more environmentally relevant conditions will need to be determined in order to assess the actual risk of HMF to the aquatic ecosystem in the case of a large spill.

Although HMF is potentially easy degradable, its toxicity to invertebrates is much higher compared to some compounds in conventional plastic production. The acute EC_{50} of TPA to *D. magna* is for example more than 1000 mg/l (OECD, 2001), approximately 20 times higher than the acute EC_{50} of HMF. For the soil invertebrate *F. candida* the toxicity of HMF is twice as high as TPA (Chen et al., 2016). These data emphasize that bio-based compounds, although derived from biomass, are not inherently safer than petroleum-based compounds, confirming the need for ecotoxicological research on these emerging compounds.

3.2. HMF induced transcriptional responses

In order to elucidate the mechanism underlying the observed toxicity, transcriptome-wide effects of exposure to HMF were assessed in an experiment in which ten-day old animals were exposed for two days to HMF in a dose range between 2.4 and 33.2 mg HMF/l. After the exposure, the animals were sampled and the extracted mRNA was sequenced using next-generation sequencing. Illumina HiSeq sequencing generated 252 million read pairs. Trimming of low-quality reads and overrepresented sequences removed on average 0.70% (±SE 0.002) of the reads. Of the remaining reads, 93.5% (±SE 0.002) was successfully aligned to the reference transcriptome, consisting of 54,349 assembled transcripts. The average library size per sample was 16.9 million reads (±SE 2.7) with one notable outlier (Figure S-1). The general expression pattern of this outlier was, however, not different to the other samples from the same treatment (Fig. 2A). After removal of

genes that had less than one count per million reads in two or less samples, in total 28,663 genes remained. This number of genes is comparable to that found in the *Daphnia pulex genome* (30,907) (Colbourne et al 2011) and the number of transcripts in other available transcriptomes of *D. magna* (between 26,508 and 28,187) (Orsini et al 2016). These remaining genes were subjected to differential gene expression analysis and GO enrichment analysis per treatment group. A clear dose-related pattern was observed for the number of significantly regulated genes (Table 1, Figure S-2). In total 1807 different genes were regulated among all treatments (Tables S–4), yet there were only 32 genes that were all regulated in the three highest treatments (Fig. 2C). Multivariate analysis and clustering analysis showed that the different treatment groups show a distinct gene expression pattern (Fig. 2A, Figure S-3). The gene expression patterns of the three lowest concentrations were more similar to each other than to the highest concentration, whereas the gene expression patterns at the LOEC (5.8 mg/l) were more similar to the patterns at the EC_{50} (17.2 mg/l) than to the NOEC (2.4 mg/l) (Figure S-3).

Transcriptional responses of individual genes do not necessarily provide much insight into the mechanisms of toxicity. Analysis of affected pathways or biological processes, however, can be more helpful to obtain mechanistic information. For this purpose, the significantly affected genes at the three highest concentrations were subjected to gene enrichment analysis using 'TopGO'. At the two highest concentrations, significantly enriched processes were furthermore subjected to REVIGO analysis in order to reduce redundancy.

At the LOEC (5.8 mg/l), gene enrichment analysis yielded 10 significant GO terms (Tables S–5). Notably, four of these GO terms were involved in the metabolism and degradation of sugars and polysaccharides. In addition, three of the terms were involved in hormone metabolism. Other significant GO terms found were related to superoxide metabolism, sulfidation and phenol-containing compound metabolism. Each significant term, however, was linked to only one specific gene and thus interpretation of these GO terms should be done with care. Nonetheless, the transcriptomic profile shows a clear signal of affected energy metabolism and oxidative stress metabolism.

At the EC_{50}, 70 GO terms were significantly enriched based on 331 significantly expressed genes (Tables S–5). REVIGO analysis yielded 24 significant non-redundant GO terms. Also at the EC_{50}, processes linked to energy metabolism were affected. Five of these terms were linked to sugar and polysaccharide metabolism (Figure S-4). In addition, we found three terms linked to fatty-acid metabolism. Other processes that were regulated were linked to immune responses (2 terms), mitochondrial DNA replication and stress responses (3 terms). Notable other processes affected were linked to cell differentiation, body morphogenesis, red blood cell homeostasis and vesical mediated transport between ER to Golgi.

The highest HMF concentration induced the strongest transcriptional response with in total 1638 significantly regulated genes; 331 biological processes were significantly enriched, which was reduced to 55 non-redundant GO terms using REVIGO analysis.

Table 1
Number of significantly up, down and non-regulated genes and the total number of genes per treatment in *Daphnia magna* exposed to HMF.

Concentration (mg/l)	Downregulated	Non-regulated	Upregulated	Total
2.4 (NOEC)	0	28663	0	28663
5.8 (LOEC)	39	28606	18	28663
17.2 (EC_{50})	110	28332	221	28663
33.2	926	27025	712	28663

At this concentration, lipid metabolism appeared to be strongly affected with six GO terms directly linked to this process (Figure S-5). Unlike the transcriptional responses in lower treatments, at the highest test concentration a clear signature of an altered stress metabolism is notable. Biological processes linked to stress metabolism included among others, response to fungicide, response to X-ray and response to mercury ion. In addition, we found three terms that were linked to DNA repair. Ten terms were linked to amino-acid or protein modification, possibly reflecting regulation of stress responses through post-translational modification (Kültz, 2005). Other affected processes were linked to vitamin metabolism (2 terms), excretion (2 terms), mitochondria formation and extracellular organization (2 terms). In addition, two terms were related to sexual reproduction. Although animals at the start of the experiment were of the same age and likely to be on the same position of the reproduction cycle, exposure to HMF at high doses might lead to the reproduction cycle to be stalled or slowed down. In fact, reproduction was delayed by 1.3 days already at a concentration of 13.8 mg HMF/l (Tables S–2). Accordingly, the transcriptome signal of those animals might thus reflect not only direct physiological stress but also changes in the reproduction cycle of the exposed animals. The limited induction of *a priori* expected pathways (see below), especially at higher doses, might be linked to non-specific changes in the metabolism including reproduction cycle. Although direct overlap in the regulated genes and processes between different treatment is limited (Figure S-6), sugar-, polysaccharide-, and fatty-acids or lipid metabolism was identified in all of the three highest HMF exposure concentrations. This response could be linked to an HMF-induced alteration of the energy metabolism. Under stress, animals may alter their energy metabolism in order to maintain cellular homeostasis or to activate stress responses (Sokolova et al., 2012). In fact, altered energy metabolism is considered as part of the evolutionary conserved cellular stress response (Kültz, 2005). Similar responses were also found in daphnids and other crustaceans exposed to an anti-cancer drug (Borgatta et al., 2015), a flame retardant (Giraudo et al., 2015), an insecticide, and a herbicide (Lee et al., 2015), which suggest that alteration of energy metabolism is a common stress response also in crustaceans. Nonetheless, the limited overlap between treatments in affected biological processes suggests that transcriptional responses are rather dose-specific limiting the possibility to identify modes of action of the toxicants (Van Straalen and Feder, 2012).

3.3. Limited induction of the xenobiotic metabolic and oxidative stress pathway

An important group of enzymes involved in the metabolism of xenobiotics is cytochrome p450 monooxygenase (CYP). These CYP enzymes are involved in oxidation reactions through hydrolysis of xenobiotic compounds leading to more hydrophilic metabolites. The metabolites can subsequently undergo reactions with conjugating enzymes, also called phase II metabolism (Anzenbacher and Anzenbacherová, 2001). These conjugates can then be excreted from the cell by ATP binding cassette transporters (ABC transporters) during phase III metabolism. These systems are widely conserved among the tree of life and are also present in crustaceans including daphnids. Baldwin et al. (2009) analyzed the CYP superfamily using the *D. pulex* genome and found 75 functional CYPs and three pseudogenes, divided over four CYP lineages. Also in daphnids and other crustaceans, phase I and phase II enzymes are responsive to xenobiotic compounds and involved in detoxification of xenobiotics (David et al., 2003; Hook et al., 2014; Ikenaka et al., 2007, 2006; Kashian, 2004; Koenig et al., 2012).

In order to test the involvement of phase I and phase II enzymes in the detoxification of HMF, we manually searched for significantly

regulated genes annotated to be involved in xenobiotic and oxidative stress metabolism (Tables S–6). We found 32 genes that were annotated as cytochrome p450 enzymes, belonging to CYP families 2, 3 and 4. Most regulated genes were downregulated but often only significantly at the highest concentration. Only seven genes were marginally upregulated, but also here, often only significantly at the highest concentration. The CYP-family comprises a wide group of enzymes involved in different processes including xenobiotic metabolism but also hormone and fatty acid metabolism (Anzenbacher and Anzenbacherová, 2001). Because the CYP family in daphnids or crustaceans is less well studied and defined in comparison to insects and vertebrates, it was not possible to determine exactly which genes are involved in which process. However, the downregulation or minimal upregulation of almost all CYP genes found in this study suggests that HMF exposure only mildly affects phase I xenobiotic metabolism. This is in contrast to the responses observed in daphnids exposed to other xenobiotic compounds, including polyphenol tannic acid (David et al., 2003), bifenthrin (Hook et al., 2014) and toxaphene (Kashian, 2004). On the other hand, lack of a clear induction of phase I metabolism in response to xenobiotic exposure has also been reported by other authors (Borgatta et al., 2015; Giraudo et al., 2015; Pereira et al., 2010).

In total 22 genes involved in phase II metabolism of xenobiotics were found to be regulated. Most of these genes were annotated as sulfotransferases, an important phase II enzyme involved in the metabolism of xenobiotics (Table S6). One gene encoding for glutathione transferase, another enzyme involved in the phase II metabolism, was also found to be regulated. As with phase I metabolism, most genes were downregulated or marginally upregulated but often only at the highest HMF concentration. A similar pattern was found for genes involved in oxidative stress. Four genes encoding enzymes associated with the metabolism of alpha-tocopherol and two genes encoding a superoxidase dismutase were downregulated whereas only one gene annotated as glutathione peroxidase was marginally upregulated.

Although some genes involved in xenobiotic or oxidative stress metabolism were upregulated in response to HMF, most of these genes were only expressed at the highest test concentrations. The lack of a clear dose-related response of CYP enzymes suggests that phase I metabolism is less important for the detoxification and excretion of HMF. Due to its aldehyde and alcohol group, the solubility in water of HMF is high (log $P_{ow} = -0.09$) (National Library of Medicine (US), n.d.). Increasing solubility by adding hydroxyl groups to this compound therefore may not be required for its excretion, especially in an aqueous environment. Another possible explanation for their limited expression is that background levels of CYPs and phase II enzymes are high enough to enable sufficient excretion of HMF. However, this explanation seems unlikely as CYP genes are expressed at log counts per million levels between one and two, which is extremely low. Moreover, the metabolism of xenobiotic compounds may also lead to more reactive compounds (Dekant, 2009). HMF can be bio-converted into 5-sulphoxymethylfurfural (SMF) (Husøy et al., 2008; Surh and Tannenbaum, 1994), which is far more reactive than HMF and was shown to have mutagenic and carcinogenic properties in mammalian cells (Monien et al., 2012, 2009). Downregulation of the sulfotransferases, as observed in this study, might therefore be a response to decrease the production of reactive compounds like SMF. Lastly, a possible alternative detoxification route for HMF might be through aspecific dehydrogenase of the alcohol group. In bacteria, aspecific alcohol dehydrogenases are involved in the biotransformation of HMF (Wierckx et al., 2011). In fact, several genes encoding for alcohol dehydrogenase were upregulated in a dose dependent manner, but only significantly regulated at either EC_{50} or highest test concentration. No aldehyde dehydrogenases were significantly regulated, suggesting that aldehyde groups are not bio-transformed.

3.4. Toxicant dose-responsive candidate genes

For the development of gene-expression based bioassays, it is essential to find genes that show a dose-related response and are consistently regulated upon exposure to a toxicant. In this study, we found thirty-two genes that were all regulated in the three highest HMF concentrations: thirteen were upregulated and nineteen downregulated (Fig. 2C; Tables S–6). Using blastx, twenty-six genes were successfully re-annotated. Genes significantly regulated included a homologue to apolipophorin (a protein involved in lipid transport), bullous pemphigoid antigen I (an antigen linked to a human skin disease) and microtubule-actin crosslinking factor 1 (a protein able to from bridges between cytoskeletal elements). Other genes were linked to cell wall associated hydrolase and titin, a protein involved in passive elasticity of muscles. Interestingly, nine genes were annotated as hemoglobin. Only four out of the thirty-two genes were upregulated with a log fold change higher than two. One of these genes could not be annotated, the other three genes encoded for hemoglobin (Fig. 3) (Tables S–6).

D. magna is well known for its ability to increase its hemoglobin concentrations rapidly in response to low oxygen levels (Gorr et al., 2004). This ability provides daphnids a high tolerance to hypoxia and allows animals to sustain aerobic metabolism under oxygen-limited conditions (Pirow et al., 2001). Ten almost identical hemoglobin genes were found in the *D. pulex* genome and at least seven in the *D. magna* genome (Colbourne et al., 2011). The high number of hemoglobin genes could allow rapid production of high levels of hemoglobin and reflects the ecological significance of hypoxia tolerance for this group of species. The overrepresentation of upregulated hemoglobin genes in response to HMF exposure, as observed in this study, could also indicate a general role of hemoglobin in the response to chemical stressors. In fact, upregulation of hemoglobin genes is frequently reported and has been observed in response to various chemical stressors including flame retardants (Giraudo et al., 2015), herbicides (Pereira et al., 2010; Rider and LeBlanc, 2006), inorganic nanoparticles (Rainville et al., 2014) and metal ions (Ha and Choi, 2009). Accordingly, the hemoglobin response has been suggested as a potential biomarkers indicative for general stress response in daphnids (Ha and Choi, 2009). Under low-oxygen levels, hemoglobin gene expression in daphnids is regulated by hypoxia inducible factors (*hif*) (Gorr et al., 2004). However, hemoglobin gene expression is additionally regulated by the terpenoid hormone methyl farnesoate, the crustacean equivalent to the insect juvenile hormone (Rider et al., 2005). In this study, a gene annotated as juvenile hormone epoxide hydrolase, an enzyme regulating juvenile hormone in insects, was upregulated. Additionally, we found eight and fifteen *egn1* genes, also known as the hypoxia inducible factor *hif*, upregulated at the EC_{50} and the highest test concentration, respectively. Based on these results, both pathways might be involved in the hemoglobin response. Mixture modelling may be required to determine through which pathway hemoglobin expression is induced under HMF exposure (Rider and LeBlanc, 2006). Nonetheless, the sensitive and dose-dependent expression response of hemoglobin genes as reported in this study provides further support for the use of hemoglobin gene expression in daphnids as a biomarker for stress.

3.5. Comparative transcriptomics

Extrapolation of toxic response observed in a single or few model species to a broader range of species in an ecosystem is a

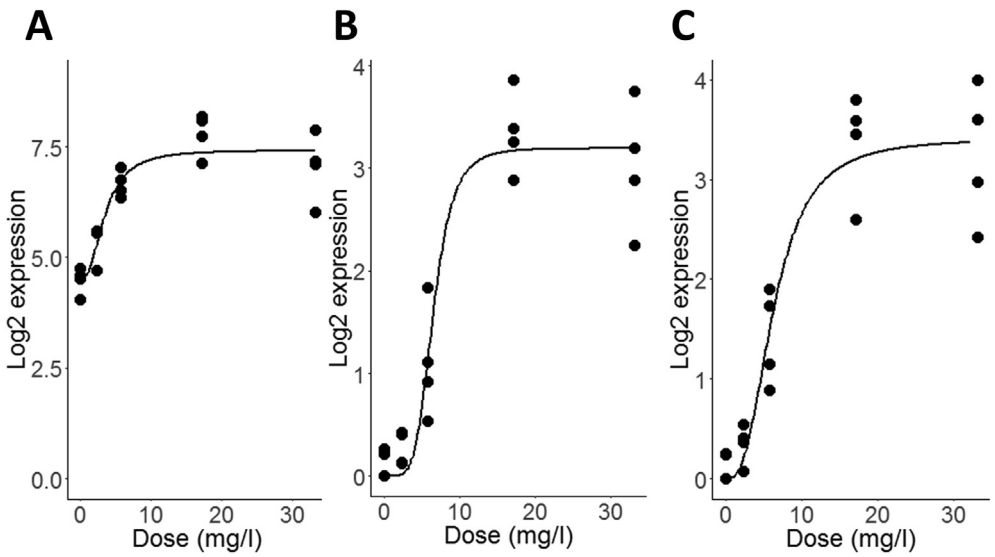

Fig. 3. Log2-transformed normalized expression values of three hemoglobin genes in *Daphnia magna* exposed to HMF: (A) c19339_g3_i1, (B) c25962_g1_i1, (C) c25962_g2_i2. Gene codes match to Tables S–6. Dots indicate Log2 normalized expression per sample. Line indicates log-logistic model.

crucial aspect in environmental risk assessment. The adverse outcome pathway framework (AOP) has been regarded as an approach to support cross-species extrapolation in a mechanistic context (Celander et al., 2011). Crucial for an AOP-based cross-species extrapolation is, however, the identification of evolutionary conserved stress pathways. Comparative transcriptomics can be used to identify such conserved pathways (Groh et al., 2015). Here, we compared the transcriptomic responses induced by HMF in *D. magna* and *F. candida* by identifying orthologous genes. Both species were exposed to equal levels of toxicity being a concentration causing 50% reduction in reproduction. In contrast to the transcriptomic responses in *D. magna* (discussed above), a strong signal of xenobiotic and oxidative stress metabolism was noticeable in *F. candida* (Chen et al., 2016). We found 4189 orthologue genes between *D. magna* and *F. candida*. In *D. magna* thirty-two out of the 4189 orthologue genes were significantly upregulated and twelve were significantly downregulated (Tables S–7). For *F. candida*, 1266 orthologue genes were significantly upregulated and 316 were significantly downregulated. Most of the orthologue genes were however, not co-regulated (i.e. significantly regulated in the same direction) in both species. In order to study transcriptional pattern in both species, multivariate analysis of the orthologue gene set was done. Fig. 4 shows the first two axis of the principal component analysis. The different sample groups (i.e. *D. magna*-control, *D. magna*-HMF, *F. candida*-control and *F. candida*-HMF) clustered separately on the first two axes and were significantly different based on their ordination on all axes ($F(3,10) = 1.71$, $p < 0.001$). Species and treatment could, however, not explain the transcriptional patterns ($F(2,11) = 0.89$, $p = 0.66$). In fact, only on the third ordination axis, exposure to HMF induced a shift in the ordination on the same axis in the same direction (Figure S-7). The limited number of co-regulated genes and the lack of explanatory power on the transcriptional responses implies that HMF induces different mechanistic responses in both test species. This can best be observed in Fig. 4 where on the first principal component axis, upon exposure to HMF, the ordination of both *D. magna* and *F. candida* shifts along the first axis but in opposite directions. In other words, genes upregulated in one species are most probably downregulated in the other and vice versa.

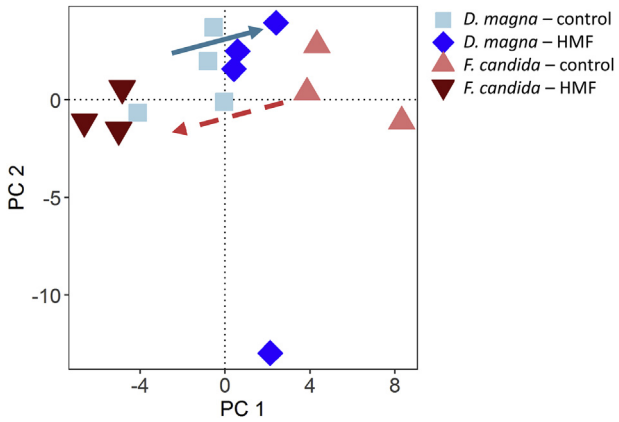

Fig. 4. First two axis of the principal component analysis of the standardized expression values at EC_{50} levels. Arrows indicate the direction of change in gene expression patterns from control to HMF for *Daphnia magna* (blue arrow) and *Folsomia candida* (red dashed arrow). The proportion of variance explained by PC1 and PC2 equals 0.24 and 0.14 respectively. Different points indicate ordination of the different samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Only a very small subset of all orthologue genes was co-regulated. We found fourteen orthologues genes that were upregulated in both *D. magna* and *F. candida* whereas only seven genes were downregulated in both species. Upregulated genes in both species included a gene that was annotated as prolyl 4-hydroxylase subunit alpha 1 (*ph4a1_rat*), which encodes for a component of an enzyme involved in the synthesis of collagen (a structural protein found in many connective tissues. DNA topoisomerase III beta 1 (*top3b_hum*) was also significantly upregulated in both species. This gene encodes for DNA topoisomerase which is involved in transcriptional activation by unfolding DNA double helix to allow access by RNA polymerase complex. Furthermore, HMF exposure at EC_{50} levels induced in both species upregulation of genes involved in oxygen homeostasis namely *egln1-mouse* and *egln3_rat*, both also known as hypoxia inducible factor (*hif*). Downregulated genes in both species included *sls*, a gene that in invertebrates encodes for

a muscle elastic protein. *Vit-6* was also found to be downregulated in both species. This gene encodes for vitellogenin-6 which in nematodes is involved in the transport and storage of lipids possibly providing resistance to environmental stress such as bacteria. Moreover, *syne1*, a gene in humans possibly involved in cytokinesis organization, was downregulated in both species.

The data presented here shows distinct differences in the transcriptomic responses under HMF exposure between a crustacean and a collembolan dosed at a similar level based on effects on reproduction. Collembola (Hexapoda; Apterygota) are a direct sister group of the insects and can be seen as taking an evolutionary transitionary position between Crustacea and Insecta (Timmermans et al., 2008). Comparative studies with insects and crustaceans have highlighted the many physiological and morphological traits including basal morphology, physiological responses to hypoxia (Harrison, 2015) and immune system functioning (Burnett and Burnett, 2015; Hillyer, 2015) shared by the two groups. However, notable anatomical and physiological differences in for example exoskeleton and nitrogen excretion also exists (Van Roer et al., 2015; Weihrauch and O'Donnell, 2015). These physiological and anatomical differences could reflect adaptation to terrestrial vs aquatic environments (Tamone and Harrison, 2015). Comparative transcriptomics between springtails, insects and crustaceans has identified several genes related to energy metabolism that have been subject to accelerated evolution in the terrestrial lineage (Faddeeva-Vakhrusheva et al., 2017). Comparative stress physiology is less well studied, but differences in physiological responses to environmental stressors like xenobiotics, as observed in this study, might also reflect different environments requiring different strategies to deal with stressors. The data presented here shows that extrapolation of stress responses among different taxonomical groups is not always possible, which may limit the possibility to identify universal markers of stress.

4. Conclusions

The transition towards a bio-based chemical industry will mean that the environment will be increasingly exposed to green chemicals. As bio-based chemicals are not inherently safer than conventional chemicals, sufficient ecotoxicological testing of novel compounds will be required to protect human health and the environment. In this study we provide data on the toxicity of a green chemical building block, which may guide future design and production of green chemistry compounds. Although HMF exposure has a strong impact on the daphnid transcriptome, daphnids do not seems to activate xenobiotic or oxidative stress metabolism, possibly due the high solubility of the compound. The limited overlap between the transcriptional responses in *F. candida* and *D. magna* exposed to HMF at a similar dose, indicates limited conservation of stress responses among soil and aquatic invertebrates. Hemoglobin transcriptional regulation suggests dose-dependency to chemical stressors and may provide a genetic marker for measuring more general adverse effects in daphnids.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.05.057.

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Declaration of interest

None.

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